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Effect of *p*H on the Denaturation of β-Lactoglobulin and its Dodecyl Sulfate Derivative

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Factors that affect the stability of β-lactoglobulin are evaluated. It denatures unimolecularly in buffer solutions more alkaline than *p*H 8. As indicated by increase in optical rotation, the rate of denaturation of β-lactoglobulin is inversely proportional to the 1.1 power of the hydrogen ion concentration at 3 and 25° in both veronal and borate buffers. Under similar conditions, β-lactoglobulin that contains two equivalents of dodecyl sulfate denatures more slowly than does normal β-lactoglobulin and shows a positive temperature coefficient of denaturation. The effects of *p*H on denaturation of β-lactoglobulin are only partially consistent with the idea that denaturation is associated with ionization of amino groups. Denaturation of β-lactoglobulin by *p*H, heat, urea, guanidine hydrochloride and a detergent is briefly compared.

The stability of β-lactoglobulin solutions is of importance in its preparation and characterization. Bull[2] noted that when it is dissolved with dilute sodium hydroxide during recrystallization, its sensitivity to local excess of alkali causes a formation of a heterogeneous product. Its denaturation, particularly by urea, has been extensively studied.[3,4,5,6] Also it has been found[6] that denaturation of β-lactoglobulin is accompanied by a large change in optical rotation and that the rate is slow at *p*H 8.25, but increases at higher *p*H values.

Our previous work[7] on the heat stability of β-lactoglobulin and its dodecyl sulfate derivative, in dilute salt solutions at *p*H 5.2, demonstrated that the presence of two molecules of dodecyl sulfate attached to the β-lactoglobulin molecule increases its stability. The present communication describes the rate of denaturation of solutions of β-lactoglobulin and its dodecyl sulfate derivative as a function of *p*H at 3 and 25°

Materials and Methods

β-Lactoglobulin and β-Lactoglobulin Dodecyl Sulfate.—These substances were prepared and purified as previously described.[7] For denaturation studies, approximately 2% solutions were prepared in veronal or borate buffers of 0.1 ionic strength and of different *p*H. Protein concentrations were determined by dry weight at 105° as well as by the Nesslerization method for determining nitrogen before diluting to 2% in the buffers.

Denaturation was measured by optical rotation and by insolubility at the isoelectric point in the presence of salt. In earlier experiments, toluene was used as a preservative. It was found, however, that turbidity, caused by toluene complicated determination of optical rotation and that essentially the same values were obtained without a preservative. Consequently most of the measurements were made on solutions without preservatives. The protein solutions were kept in polarimeter tubes, and all readings of optical rotation were made at 25°. The solutions kept at 3° were warmed rapidly by immersing the polarimeter tube in water at 25°. After the readings were taken, the solutions were immediately placed in the water-bath at 3°. The specific rotation of undenatured and denatured protein varies with *p*H, and consequently it was necessary to know the specific rotation of the undenatured and denatured protein as a function of *p*H. Since the extent of denaturation is a function of time, a method was needed for determining the specific rotation of native proteins at zero time for each *p*H value. The value for specific rotation at zero time has been estimated by taking readings immediately after adjusting the protein solutions to the desired *p*H, as well as by plotting log $(\alpha_t - \alpha_\infty)$ against time and extrapolating to zero time.[8] The results obtained by the two methods generally were in agreement. The specific rotation of denatured protein was calculated from the maximum specific rotation values in the higher *p*H ranges, where denaturation went to completion. Specific rotation values as a function of *p*H were also determined on isolated completely denatured protein in the presence of veronal buffer of 0.1 ionic strength. Figure 1 summarizes the effect of *p*H on the specific rotation of undenatured and denatured β-lactoglobulin and its dodecyl sulfate derivative.

In determining denaturation by insolubility at the isoelectric point, the denatured protein was precipitated by adding 0.1 *N* acetic acid to *p*H 5.0. The insoluble protein was removed by centrifugation and washed with 0.05 *M* sodium chloride. The protein was estimated from the nitrogen content of the insoluble material as determined by the Nesslerization method, the conversion factor of 6.40 was used.

The accuracy of the two methods was evaluated on known mixtures of native and denatured β-lactoglobulin. These mixtures were made to a total concentration of 2% protein at *p*H 8.44 in veronal buffer. The results obtained for the amount of denatured protein in the mixture by the two methods were in good agreement with the known composition. The values obtained by insolubility at the isoelectric point were slightly more precise than those found by the optical rotation method on the unseparated mixture. The

(1) One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Article not copyrighted.

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(4) K. Linderstrøm-Lang, *Cold Spring Harbor Symposia Quant. Biol.*, **14**, 117 (1949).

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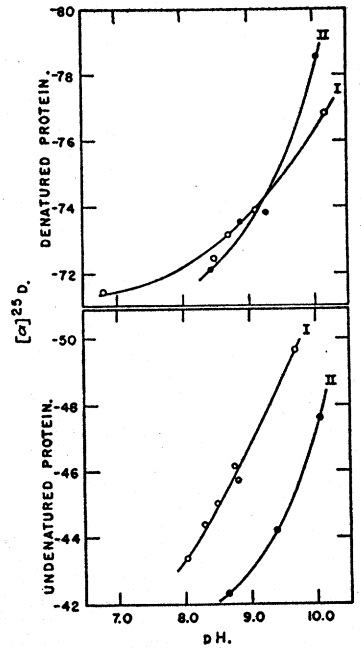

Fig. 1.—Effect of *p*H on the specific rotation of undenatured and denatured β-lactoglobulin and its dodecyl sulfate derivative: I, β-lactoglobulin; II, β-lactoglobulin dodecyl sulfate.

convenience of the optical rotation method is greater, however, since no manipulation is required to follow the rate of denaturation. Consequently, the results herein reported were obtained by the optical rotation method unless otherwise stated.

The determinations of *p*H were made with the glass electrode. Potassium acid phthalate and borate buffers were used in standardizing the instrument. Measurements of *p*H were reproducible to within 0.04 unit.

The *p*H values of the β-lactoglobulin solutions at 3° in both veronal and borate buffers became more alkaline on changing from 25 to 3°. In the protein solutions in borate, the shift in *p*H with temperature was in agreement with the values interpolated from the data of Walbum[9] extrapolated to 0°. It appears to be common practice to consider the *p*H change of veronal buffers with temperature to be negligible.[10] The *p*H values for 3° given in the text were based on measurements at 3°.

Results

The results of individual rate measurements on the effect of *p*H and temperature on denaturation of β-lactoglobulin and its dodecyl sulfate derivative are given in Figs. 2 and 3 as the log of the per cent. undenatured. It is impractical to give all the details regarding the data. However, all the specific rotation measurements may be derived from Figs. 2 and 3 by means of the relation $100/\%$ undenatured $= (\alpha_0 - \alpha_\infty)/(\alpha_t - \alpha_\infty)$. The undenatured protein at time t is read from Fig. 2 or 3. Values for α_0 and α_∞ are obtained from Fig. 1 as a function of *p*H. A value of -51.5 for α_0 for β-lactoglobulin at *p*H 10.1 obtained by plotting log $(\alpha_t - \alpha_\infty)$ against time and extrapolating to zero time was used instead of the value of -49.7 as given in Fig. 1, since it made possible more consistent interpretation of the results.

The fact that the logarithm of the concentration of the undenatured protein plotted against time is a straight line indicates that denaturation is a unimolecular reaction. The velocity constant k has been calculated by the unimolecular formula $k = 2.303/t \log C_0/C$, where C_0 represents the concentration at the beginning of the experiment and is equal to 100, and C represents the per cent. concentration of undenatured protein after time t, as shown in Figs. 2 and 3.

Figure 4 shows the relationship of the logarithm of the velocity constant k to *p*H for both β-lactoglobulin and its dodecyl sulfate derivative. The method of least squares was used in drawing curve 1 of Fig. 4. For a given *p*H, the rate of denaturation of β-lactoglobulin is much greater than that of its dodecyl sulfate derivative. The fact that the rate of denaturation of β-lactoglobulin is the same at 3° as at 25° is difficult to explain. The rate is inversely proportional to the 1.1 power of the hydrogen ion concentration at 3 and 25° in both veronal and borate buffers. The effect of temperature on the rate of denaturation of the dodecyl derivative is normal, being greater at 25° than at 3°

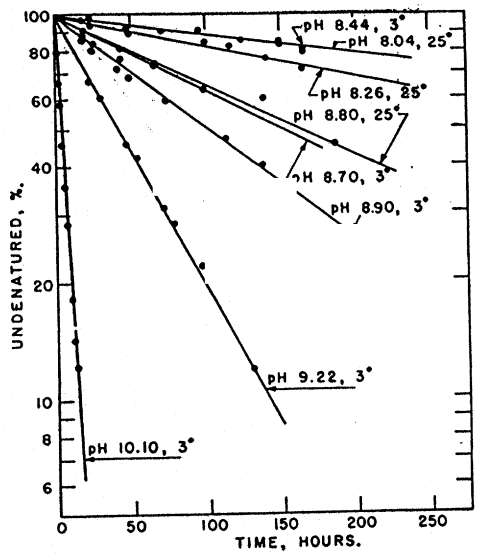

Fig. 2.—Effect of *p*H on the velocity of denaturation of β lactoglobulin in veronal buffer at 3 and 25°

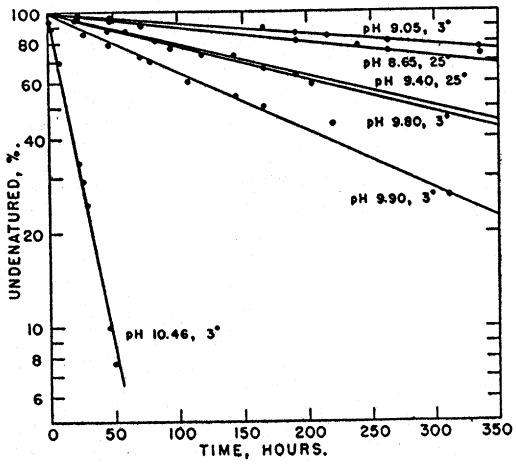

Fig. 3.—Effect of *p*H on the velocity of denaturation of β-lactoglobulindodecyl sulfate derivative in veronal buffer at 3 and 25°.

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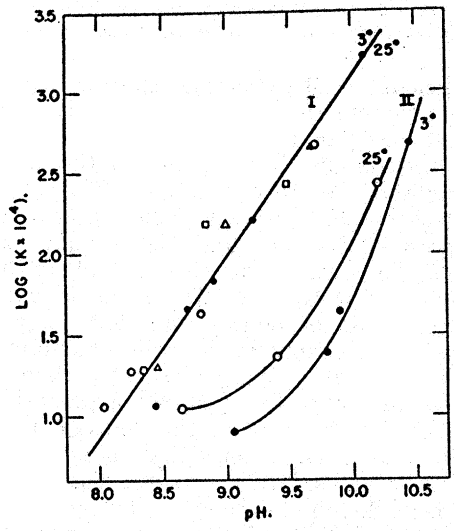

Fig. 4.—Comparison of the effects of *p*H and temperature on the velocity-constant of denaturation: I, β-lactoglobulin; II, β-lactoglobulin dodecyl sulfate; □, borate at 25°, △, borate at 3°, O, veronal at 25°, ●, veronal at 3°.

At *p*H 8.04 and 10.10, values for the velocity constant of denaturation of β-lactoglobulin in veronal buffer determined by insolubility at the isoelectric point agree with those obtained by optical rotation. At intermediate *p*H values, however, the velocity constants obtained by measurements of insoluble protein were less than those obtained by optical rotation, as shown in Fig. 5.

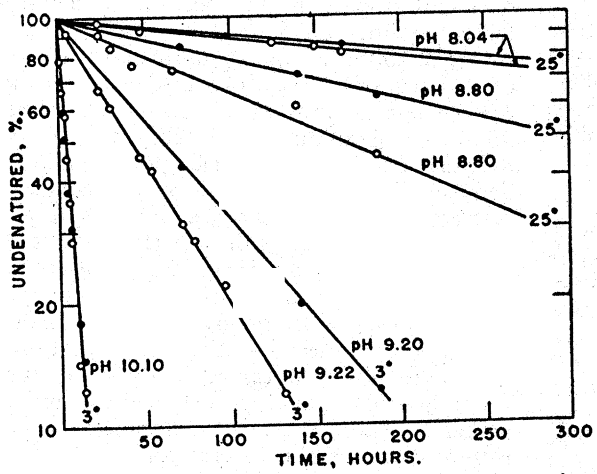

Fig. 5.—Comparison of the rate of denaturation of β-lactoglobulin in veronal buffer as determined by optical rotation (O), and insolubility at the isoelectric point in the presence of salt (●).

In borate buffer, the rate of denaturation as determined by insolubility at the isoelectric point was in good agreement with the value obtained by optical rotation changes at each *p*H value studied.

Sulfhydryl Groups in Denatured β-Lactoglobulin.—An attempt was made to follow the rate of appearance of sulfhydryl groups during *p*H denaturation by the nitroprusside test. The color produced, however, was too feeble to be estimated even in the completely *p*H denatured protein. Solutions of β-lactoglobulin heated at 80° for 20 minutes in veronal buffer gave 0.15% of cysteine by the porphyrindin method.[11] A slightly lower value of 0.14% was found for the dodecyl sulfate derivatives under similar conditions. In 5 *M* guanidine hydrochloride, the apparent amount of sulfhydryl in β-lactoglobulin was increased to 0.55% cysteine. This value is in good agreement with the value of 0.5 sulfhydryl groups per 10^4 grams protein reported by Jane Fraenkel-Conrat,[12] and one-half the amount reported by Brand, *et al.*,[13] for the cysteine content of acid hydrolyzed β-lactoglobulin.

Mobility of Denatured β-Lactoglobulin.—Because of differences in method of separating the denatured protein, the electrophoretic mobility and homogeneity of *p*H denatured β-lactoglobulin appears to vary. Thus when it is denatured at *p*H 10.1 at 3° the denatured protein separated by adjusting to *p*H 5.2 is electrophoretically homogeneous and has a mobility of 5.37 u (u = cm.^{2} volt^{-1} sec.$^{-1}$ × 10^5) in veronal buffer of *p*H 8.4 and 0.1 ionic strength. This mobility is slightly greater than the value of normal β-lactoglobulin of 5.10–5.20 under the same conditions. Somewhat different electrophoretic results were obtained on *p*H denatured β-lactoglobulin denatured in the same way but dialyzed before being adjusted to the isoelectric point, as well as on denatured protein which had been redissolved and reprecipitated. In these instances, the products had two electrophoretic components, one of which had a mobility of about 5.2–5.3 and the other 5.9–6.3 u. β-Lactoglobulin denatured with guanidine hydrochloride was electrophoretically homogeneous and had a mobility of 5.52 u. Variations in electrophoretic homogeneity and mobility with the method of purification indicate that denatured β-lactoglobulin is very reactive, producing components of different electrophoretic properties. This finding is in qualitative agreement with the results reported by Briggs[14] for heat-denatured β-lactoglobulin. β-Lactoglobulin dodecyl sulfate derivative denatured in buffer at *p*H 10.7 had two electrophoretic components, one of which had a mobility of 5.92 u, being the same as the undenatured material, whereas the other component had the slightly greater mobility of 6.33 u.

Crystalline β-lactoglobulin recovered from the filtrates of incompletely denatured β-lactoglobulin by *p*H or by guanidine hydrochloride had the same electrophoretic composition and mobility at *p*H 4.7 as normal β-lactoglobulin, showing that neither of the two electrophoretic components[15] of β-lactoglobulin is preferentially denatured.

Other Denaturing Agents.—The specific rotations of denatured β-lactoglobulin denatured by *p*H, heat, dodecyl sulfate, urea and guanidine hydrochloride are given in Table I. The low value for the specific rotation of dodecyl sulfate

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TABLE I

EFFECTS OF VARIOUS DENATURING AGENTS ON THE OPTICAL ROTATION OF β-LACTOGLOBULIN IN 2% SOLUTIONS

Denaturing agent		$[\alpha]^{25}D$	$[\alpha]^{25}D$[a]
*p*H 8.55		− 72.9	
Heat (80°) at *p*H 8.55		− 79.7	
0.17 *M* dodecyl sulfate	initial	− 59.6	
	final	− 59.6	
6.6 *M* urea	initial	− 63.0	
	final	−107.0	−75.0 (*p*H 8.3)
5 *M* guanidine hydrochloride	initial	−114.4	
	final	−114.4	−69.2 (*p*H 7.2)

[a] After removal of denaturant.

denatured β-lactoglobulin is noteworthy. This protein was completely denatured, as indicated by insolubility at the isoelectric point.

If measured by the increase in optical rotation, the denaturation of β-lactoglobulin is practically instantaneous in 5 *M* guanidine hydrochloride. If, however, the amount of insoluble material at *p*H 5.2 after the removal of guanidine is taken as a measure of denaturation, a different result is obtained. Thus, after 2 hours standing at room temperature in 5 *M* guanidine hydrochloride, approximately 20% of the protein was insoluble after the removal of guanidine hydrochloride; after 24 hours standing, 40% became insoluble. In both cases, a crystalline protein was isolated from the soluble fraction which was identical with the original normal β-lactoglobulin in crystal form, specific rotation, solubility, electrophoretic properties and antigenic properties.[16] After exposing serum albumin to the action of concentrated guanidine hydrochloride, Neurath, *et al.*,[17] obtained a soluble and an insoluble fraction on removing the guanidine hydrochloride by dialysis. The soluble protein resembled the native protein in molecular size and shape but differed in crystallizability, electrophoretic mobility and antigenic properties. They believed that the soluble portion was formed by the reversal of denaturation of the protein.

Discussion

The influence of *p*H on the denaturation of β-lactoglobulin and its dodecyl sulfate derivative (Fig. 4) may be interpreted in terms of Steinhardt's[18] concept of the importance of ionization processes in denaturation which was recently extended by Levy and Benaglia.[19] Thus, the *p*H range of 8.0 to 9.5, where the rate of denaturation of β-lactoglobulin increases rapidly, coincides with the *p*H range where the amino groups are dissociating. That the rate of denaturation at 3° is the same as at 25° is not consistent with the ionization theory of denaturation, since ionization of amino groups at a given *p*H decreases with decrease in temperature.[20] The apparent lack of energy of activation at these temperatures is anomalous and requires explanation. In contrast, β-lactoglobulin containing two equivalents of dodecyl sulfate has a positive temperature coefficient at all *p*H values studied. Jacobsen and Christensen[5] found a large negative temperature coefficient for the early stages of denaturation of β-lactoglobulin in urea, but in the later part the coefficient became positive. The fact that its dodecyl sulfate derivative denatures in a *p*H range approximately one unit higher than does β-lactoglobulin is consistent with the shift in the titration curve of β-lactoglobulin when it contains two equivalents of dodecyl sulfate,[21] suggesting that the amino groups in the dodecyl sulfate derivative dissociate at a higher *p*H value than in β-lactoglobulin. Another possible explanation for the rapid denaturation of the dodecyl derivative between *p*H 9.4 and 10.0 is that under these conditions the dodecyl sulfate groups are dissociated from the β-lactoglobulin molecule. This possibility does not appear probable, since the electrophoretic mobility of the recovered dodecyl derivative denatured at *p*H 10.0 was 5.9–6.2 u at *p*H 8.4 in veronal buffer at 0.1 ionic strength. This indicates that the denatured protein contains two equivalents of dodecyl sulfate, since denatured β-lactoglobulin without dodecyl sulfate had a mobility of 5.3 u under these conditions.

The reports of Linderstrøm-Lang[3] on the reversal of denaturation of heat-denatured β-lactoglobulin and of Jacobsen and Christensen[5] on the reversal of denaturation of urea-denatured β-lactoglobulin indicate that the denatured β-lactoglobulin readily reverts to the native form. This does not appear to be true of *p*H-denatured β-lactoglobulin. As judged by changes in optical rotation, solubility or crystallizability no reversion of denaturation was obtained by allowing the *p*H-denatured protein to stand in dilute acid, alkali or guanidine hydrochloride followed by dialysis at the isoelectric point. However, if denaturation is determined by changes in optical rotation or the appearance of sulfhydryl groups rather than by insolubility at the isoelectric point, it is easy to demonstrate the apparent reversal of β-lactoglobulin denatured by urea or guanidine hydrochloride. On removing the urea or guanidine hydrochloride by dialysis, a portion of the β-lactoglobulin is obtained in the crystalline form which has all the characteristics of the undenatured protein. These apparent differences between the rate of denaturation in urea and guanidine hydrochloride and at some *p*H values (Fig. 5) as measured by optical rotation, by insolubility at the isoelectric point are consistent with the view that two stages are involved in denaturation. The first and most rapid stage is associated with an increase in optical rotation and the appearance of sulfhydryl groups. This stage appears to be reversible. The second stage is associated with the formation of denatured protein insoluble at the isoelectric point, which appears to be irreversibly denatured.